

STANDARD OPERATING PROCEDURE

Title: Optimizing Mass Spectrometer Performance for Experiments 1 and 2

SOP#: WU-MS1-01

Version #: 2

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1. PURPOSE

The purpose of this document is to describe the procedures for calibrating and optimizing the performance of the TripleTOF® 5600+ mass spectrometer for electrospray mass spectrometry with *nano*-LC-MS.

2. SCOPE

This procedure encompasses the i) preparation of benchmark and calibrant solutions; ii) spectral data acquisition to assess state of the instrument prior to tuning; iii) optimization procedure; and iv) assessment of instrument performance after calibration and tuning.

3. RESPONSIBILITIES

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures and the user manual for the instrument. The interpretation of results must be done by a person with expertise in mass spectrometry and familiar with such interpretation. It is the responsibility of the primary instrument operator to perform these procedures when the system has failed to meet daily specification requirements for LC-MS as given in WU-SOP-MS2-01.

4. EQUIPMENT

Source: New Objective Digital Picoview for PV-450

Emitter tip: New Objective PicoTips® emitter silica tips (FS360-20-10N-20-C20)

LC-to-source connection: Fused silica tubing (Polymicro Technologies, 1068150009)

Mass spectrometer: TripleTOF® 5600+ (Sciex)

Microcentrifuge, Eppendorf 5415D
Rainin™ Pipet-lite XLS, P20, P200, P1000

5. MATERIALS

Syringe (Hamilton 8175) (250 µL)
Fused Silica Tubing (100 µm)
Water containing 0.1% formic acid (Honeywell Burdick & Jackson, LC452-2.5)
Acetonitrile containing 0.1% formic acid (Honeywell Burdick & Jackson, LC441-2.5)
Glu-Fib (Sigma (F3261- 0.1)
Installation Kit for TTOF System™ (Cat No. 4456736, reserpine and tuning solution)
Methanol (SIGMA, 34860)
Graduated cylinders (10 mL and 100 mL)
Glass media bottle (Pyrex)
Microtubes: Axygen® MCT-175-C (311-04-051)
Axygen® MAXYmum™ recovery tips;
P200 and P20: T-200-C-L-STK,
P1000: T-1000-C-L-R

6. REAGENTS

Preparation Of Calibrant And Benchmark Solutions.

A. Preparation Of Diluent Solution (DS) (90% Water, 10% Acetonitrile, 0.1% formic acid)

- 1) Measure 90 mL of water containing 0.1% formic acid in a 100 mL graduated cylinder.
- 2) Transfer to a 100 mL glass media bottle.
- 3) Measure 10 mL of acetonitrile containing 0.1% formic acid in a 10 mL graduated cylinder.
- 4) Add to the water/formic acid solution and mix by swirling.

B. Preparation Of The Glu-Fib Solutions

- 1) Add 900 µL of DS to the Glu-Fib vendor vial (50 pmol/µL) and vortex 3 x 30s.
- 2) Rinse the microfuge tubes with methanol three times.
- 3) Dilute the stock solution by adding 180 µL of methanol to the washed microfuge tubes using a Pipetman®.
- 4) Dilute the primary stock solution by adding 100 µL to a washed tube containing 900 µL of IS and mix by vortexing as in Step 1.
- 5) Prepare two working solutions from the Glu-Fib secondary stock use the washed microfuge tubes and mix as described above (Step 1):
 - i) Glu-Fib (500 fmol/µL), 100 µL of secondary stock and 900 µL of DS;

- ii) Glu-Fib (150 fmol/ μ L), 350 μ L of secondary stock and 500 μ L of DS.

C. Preparation Of Instrument Performance Solution

- 1) Add the following quantities to a washed microfuge vial:
- 2) DS (50 μ L),
- 3) Tuning solution (50 μ L),
- 4) Reserpine (150 μ L)
- 5) Glu-Fib, 150 fmol/ μ L (150 50 μ L).

D. Preparation of Final Standards

The final concentration of standards is 75 and 56 fmol/ μ L of Glu-Fib, respectively, in a 1:10 dilution of stock tuning solution.

- This is a 1:10 dilution of Tuning Sol'n
- 56 fmol/ μ L Reserpine *m/z* 609.4
- 75 fmol/ μ L GluFib *m/z* 785.8

7. PROCEDURES

A. MS Setup Parameters

- 1) Source/Gas Parameters:
 - a) Curtain Gas (CUR): 25
 - b) IonSpray Voltage (IS): 3200
 - c) Ion Source Gas 1 (GS1): 30
 - d) Ion Source Gas 2 (GS2): 0
 - e) Interface Heater Temperature (IHT): 175°C
- 2) Scheduled PRM Parameters:
 - a) PRM detection window (sec): 600
 - b) Target Scan Time (sec): 1.5
- 3) MS Parameters:
 - a) Declustering Potential (DP): 70
 - b) Collision Energy (CE): From Skyline (default ABI TTOF 5600 algorithm)
 - c) Resolution Q1: Unit
 - d) Resolution TOF MS1: >25000
 - e) Resolution TOF MS2 >15000
 - f) Intensity threshold (total count): 50
 - g) Settling time (ms): 0
 - h) Pause between mass ranges (ms): 1.038

B. New Objective Stage Positioning and Analyst® TF Parameters

NOTE: values will vary slightly after installing a new tip. Infuse the Instrument Assessment Solution to determine the best tip position and Analyst® TF Parameters.

1) New Objective Source Stage Position

<u>Axis</u>	<u>Value</u>	<u>Range</u>
X (left-right)	10500	10,000 - 11,500
Y (in-out)	1500	FIXED
Z (up-down)	4300	4000 – 5000

2) Analyst TF® Parameters

<u>Source/Gas Tab</u>	<u>Value</u>	<u>Range</u>
GS1	3	5-15
GS2	0	Not used for nanospray
CUR	40	Fixed
ISFV	3600	3400 – 3800 typical (start new tip at 3000)
IHT	150	75 – 150

<u>Compound Tab</u>	<u>Value</u>	<u>Range</u>
DP	100	flat in 80 – 110 range; same value in TOFMS & MSMS
CE	10	FIXED in TOFMS

NOTE: Use rolling Collision Energy for IDA data acquisition.

NOTE: In most cases, parameters under the Resolution or Detector tabs should not be adjusted. These values are set by the Sciex field service engineers running the 'Instrument Optimization' function

NOTE: FIXED is based on various infusion tests with 50 fmol/ul GluFib

C. Assessing Instrument Status Prior to Optimization

- 1) Double click on Manual Tuning
- 2) Set up the following parameters:
 - a) Drop down menu: Syringe Pump Method
Diameter: 2.3 (250 µL ul syringe)
Flow: 800 nL/min
 - b) Drop down menu: MS Method
Source/Gas Tab: see above
MS tab: Duration: 1 sec
TOF Masses: min 400 max 1800
- 3) Click Start and go to drop down menu View / Graph Information Window

- 4) Zoom in on 785.8 and highlight the first peak, monitoring the TIC and adjusting the Source/Gas parameters until obtaining the highest Resolution and Intensity possible.
- 5) Click Stop.
- 6) Get a screen cap that includes the Graph Information Window and paste into Powerpoint, labeling as GluFib Baseline TOFMS PreClean/Opts
- 7) Click on Acquire

Sample Name	2014xxxx_GluFib_75 fmol_TOFMS
Data File Name	2014xxxx_GluFib_75fmol
(this goes to Analyst Data\Projects\January 2014\Data)	
- 8) Click Start
- 9) Acquire for ~2 min then click Stop
- 10) Zoom in on the TIC for about a 0.5 min range
- 11) Double click in the box, bringing up a new window
- 12) Zoom in on 785 and set threshold arrow up so it only gets the first 785 peak
- 13) RIGHT click in window and click on List Data, adding a new window
- 14) Get a screen cap, paste in Powerpoint and label as Glu Fib MS Centroid

At Spec:
 Res ≥30k Centroid Intensity: xxxx4000 (Glu-Fib)

- 15) In the MS tab:
 - a) Set Type to Product Scan
 - b) Click High Resolution
 - c) Parent mass: 785.8
 - d) Duration: 1 sec
 - e) TOF Masses: min 100 max 1800

16) In the Compound Tab: Set the Collision Energy to 45

- 17) Click on Acquire

Sample Name	2014xxxx_GluFib_66fmol_HR
Data File Name	2014xxxx_GluFib_66fmol

- 18) Click Start
- 19) Acquire for ~2 mins, then click Stop
- 20) Zoom in on the TIC for about a 0.5min range
- 21) Double click in the box, bringing up a new window
- 22) Set threshold arrow up so it only just catches the 187.07 peak
- 23) Right click in the window and click on List Data, adding a new window
- 24) Adjust the windows sizes so that the values for 187.07, 480.25, 813.39 and 1056.48 are visible. Get a screen capture, paste into PowerPoint and label as Glu Fib Product Ion – HR

At Spec:

<u>Mass</u>	<u>Resolution</u>	<u>Centroid Intensity:</u> xxxx
187.07	>20k	>40
480.25	>20k	>130

813.39	>20k	>250
1056.48	>20k	>130

25) In the MS tab: Click High Sensitivity

NOTE: no other parameters need to be changed.

26) Click on Acquire	Sample Name	2014xxxx_GluFib_75fmol_HS
	Data File Name	2014xxxx_GluFib_75fmol

27) Click Start

28) Acquire for ~2 mins, then click Stop

29) Zoom in on the TIC for about a 0.5 min range

30) Double click in the box, bringing up a new window

31) Set threshold arrow up so it only just catches the 187.07 peak

32) Right click in the window and click on List Data, adding a new window

33) Adjust the windows sizes so that the values for 187.07, 480.25, 813.39 and 1056.48 are visible.

34) Get a screen cap, paste in Powerpoint and label as Glu Fib Product Ion – HS At Spec:

<u>Mass</u>	<u>Resolution</u>	<u>Centroid Intensity</u>
187.07	>12k	>xxxx120
480.25	>12k	>425
813.39	>12k	>750
1056.48	>12k	>450

D. Instrument Optimization

1) Confirm the following parameters:

2) Drop down menu: Syringe Pump Method

- Diameter: 2.3 (250ul syringe)
- Flow: 0.8 ul/min

3) Drop down menu: MS Method

- Source/Gas Tab: 2-25
- MS tab: Duration: 1 sec
- TOF Masses: min 100 max 1800

4) Click Start and go to drop down menu View / Graph Information Window

5) Zoom in on 829.5 and highlight the first peak, monitoring the TIC and adjusting the Source/Gas parameters until obtaining the highest Resolution and Intensity possible. Click Stop.

- Resolution: ~20k Intensity: ~65k

6) Get a screen cap that includes the Graph Information Window and paste into Powerpoint, labeling as Instrument Opts Baseline TOFMS PostClean/Opts

7) Close Manual tuning and double click Instrument Optimization

8) Check all boxes, click ok

9) Enter GS1, ISFV, Syringe diameter and flow rate

10) Click OK

11) Copy the Optimization Summary Table into a Word doc

E. Post-Optimization Assessment With Glufib Standard Infusion

- 1) Repeat the Pre-Optimization GluFib Infusion, labeling all screen captures as 'Post Instr Opts'.
- 2) The instrument should meet the specifications as shown above for GluFib standard. If the instrument fails these specifications the entrance section of the instrument should be cleaned and the status again assessed as above. If instrument continues to fail specifications the mass spectrometer vendor should be consulted.

F. Procedure To Clean Q-JET

- 1) Turn the MS off.
- 2) Turn the roughing pump off
- 3) Remove nano source.
- 4) Allow source heater to cool and MS to vent
- 5) Remove source cover and heater
- 6) Remove Q-Jet and cover opening with foil
- 7) Take the lens off of the Q-Jet assembly and scrub both it and the rails with 1% Alconox
- 8) RINSE WITH TAP WATER THOROUGHLY
- 9) RINSE WITH deionized WATER THOROUGHLY
- 10) Immerse the Q-Jet in 100% Methanol, suspending it with spatulas
- 11) Sonicate for 20 mins, flip ends and sonicate for 20 mins
- 12) Blow dry with nitrogen
- 13) Re-install in instrument
- 14) Pump down overnight (green light on instrument indicates adequate vacuum has been achieved. Value can be viewed within the software.

8. REFERENCED DOCUMENTS

WU-SOP-MS2-01- Mass Spectrometry Using Parallel Reaction Monitoring for Experiments 1 and 2

9. ABBREVIATIONS

DS, instrument compatible diluent solution for preparation of benchmark solutions

STANDARD OPERATING PROCEDURE

Title: Mass Spectrometry Using Parallel Reaction Monitoring for Experiments 1 and 2

SOP#: WU-SOP-MS2-01

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1. PURPOSE

The purpose of this document is to describe the mass spectrometric (MS) methods for quantitative analysis of peptides using paired internal standards and LC-MS with parallel reaction monitoring (PRM).

2. SCOPE

This procedure encompasses the optimization of methods for a TripleTOF® 5600+ mass spectrometer that are used to execute Experiments 1 and 2 for a multiplex assay of 130 synthetic peptide pairs. The method builds and optimizations are performed using the Skyline software (<https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view>). The *nano*-liquid chromatography methods and dual column setup are described in WU-SOP-LC1-01.

3. RESPONSIBILITIES

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures and the user manual for the instruments. The interpretation of results must be done by a person with expertise in mass spectrometry and familiar with such interpretation. It is the responsibility of the primary instrument operator to perform daily monitoring of the system for meeting specifications described herein, and performing instrument evaluations as described in WU-SOP-MS1-01.

4. EQUIPMENT

Source: New Objective Digital Picoview for PV-450

Emitter tip: New Objective PicoTips® emitter silica tips (FS360-20-10N-20-C20)

LC-to-source connection: Fused silica tubing, Polymicro Technologies (1068150009)

Mass spectrometer: Sciex TripleTOF® 5600+

Micro-centrifuge: Eppendorf 5424 R
Rainin™ Pipet-lite XLS, P20, P200, P1000

5. MATERIALS

Standards, β -galactosidase tryptic digest (625 pmol) (Sciex, 4465867)
Standard peptides: QCH (WU-SOP-EXP1-1)
Autosampler vials: Sun-Sri (200 046)
Autosampler vial caps: Sun-Sri (501 382)
Clear vials (4 mL, National Scientific, B7990-2)
Axygen® MAXYmum™ recovery tips;
P200 and P20: T-200-C-L-STK,
P1000: T-1000-C-L-R

6. REAGENTS

Acetonitrile (Fluka, 34967-1L)
Formic Acid, (Fluka, 56302-50ML-F)
Water (Fluka, 39253-1L-R)

7. PROCEDURE

A. Mass Spectrometer Default Parameters (see WU-SOP-MS1-01 for optimization method)

- 1) Source/Gas Parameters:
 - a) Curtain Gas (CUR): 25
 - b) IonSpray Voltage (IS): 3200
 - c) Ion Source Gas 1 (GS1): 30
 - d) Ion Source Gas 2 (GS2): 0
 - e) Interface Heater Temperature (IHT): 175°C
- 2) Scheduled PRM Parameters:
 - a) PRM detection window (sec): 600
 - b) Target Scan Time (sec): 1.5
- 3) MS Parameters:
 - a) Declustering Potential (DP): 70
 - b) Collision Energy (CE): From Skyline (default ABI TTOF 5600 algorithm)
 - c) Resolution Q1: Unit resolution
 - d) Resolution TOF MS1: >25000
 - e) Resolution TOF MS2 >15000
 - f) Intensity threshold (total count): 100
 - g) Settling time (ms): 0
 - h) Pause between mass ranges (ms): 1.038

B. Column Conditioning

- 1) Run 102 min IDA method 10 times and inject 10 μ L of TEN-MIX-1-100 solution (preparation described in WU-SOP-EXP1-01).

C. Assessing LC-MS Performance

- 1) Preparation of test solution (β -galactosidase tryptic digest working solutions)
 - a) Add 625 μ L of 10%AcN, 0.1%FA to vendor vial to prepare a 1 pmol/ μ L primary stock solution
 - b) Vortex vigorously for 30 sec.
 - c) Spin 30 seconds at 14,000 rcf.
 - d) Dilute 1:20 with **AcN/FA-1** (described in WU-SOP-EXP1-01) in 4 mL glass vial to prepare secondary stock (50 fmol/ μ L).
 - e) Store as 200 μ L aliquots in AS vials at -20°C.
 - f) Thaw secondary stock at room temperature.
 - g) Dilute the secondary stock ten-fold with the **AcN/FA-1** solutions.
 - h) Transfer 200 μ L to each of three autosampler vials for injection of 50fmol/10 μ L by the autosampler (WU-SOP-LC1-01).

- 2) Assessing instrument performance for LC-MS with PRM mass spectrometry
 - a) LC-MS analyses with the standard β -GAL samples are opened in PeakView® to assess the total ion current trace. Files are processed in the LCMS Peak Statistics application. A report is exported for MS1 data and MS2 data to a .csv file. The .csv files are opened in Excel and the data are copied to the TTOF_performance_CURRENT Excel spreadsheet. The Excel spreadsheet plots TOFMS resolution, intensity and retention time for the 729 m/z precursor mass of a β -galactosidase tryptic peptide, and the 5 most intense transitions from the spectrum.

MS1 m/z	Transitions	Transition m/z
433.8792	y3	347.2037
450.6960	y5	563.2784
528.9341	y8	832.4523
542.2645	y10	1061.522
567.0551	y12	1289.633
607.8588	Parent	729.3652
671.3379		
729.3652		

- b) The benchmark specifications are given below:
 - TOFMS resolution >25,000
 - 729 intensity: $\geq 1e5$
 - MS2 resolution >15,000
 - 832 intensity ≥ 500

- The optimization steps and LC-trouble shooting is initiated if instrument fails to meet specifications (WU-SOP-MS1-01).

D. Identifying Scheduling Times For Targeted Peptides

- 1) Targeted LC-PRM-MS method building.
 - a) Load the Skyline file (WU-SOP-MS-2-MB1) containing peptide sequences, precursors, and transitions to be monitored during the LC-PRM-MS experiment.
 - b) In the Skyline document under Settings/Transition Settings/Predictions, select 'ABI TTOF 5600' under 'Collision energy:' and 'ABI' under 'Declustering potential'.
 - c) Export the unscheduled precursor *m/z* list as a report File/Export/Report.
 - d) Import the unscheduled precursor *m/z* list as a DDA with inclusion list (DDAi) method on the TTOF 5600+ using the Analyst® TF v1.6 (<http://sciex.com/products/software/analyst-software>) method wizard with all other instrument parameters set as above (Part 1).

- 2) Building a scheduled method.
 - a) Set up the autosampler and LC methods as described in WU-SOP-LC-1 and for either Experiment 1 (WU-SOP-EXP1-01) or Experiment 2 (WU-SOP-EXP2-01).
 - b) Inject a (10 µl) of the QC sample containing equimolar H/L standard peptide mix (20 fmol/uL) light/heavy standard peptides in target matrix and acquire the PRM-MS data. Perform acquisition on both LC columns.
 - c) Import the data files for each column into the Skyline document (3A).
 - d) Manually check the automatic peak selection/integration of all peaks. Adjust if necessary. See Skyline tutorial, "Targeted Method Refinement" for reference. https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_method_refine).
 - e) Export the scheduled precursor *m/z* list as a report in Skyline with a column for "Precursor Mz" and "Average Measured Retention Time" averaged using above scheduled parameters. See Skyline tutorial, "Custom & Live Reports" for reference. https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_custom_reports.

- 3) Scheduled method testing.
 - a) Import the scheduled precursor *m/z* list as a scheduled DDAi method on the TTOF 5600+ using the Analyst® TF method wizard with all other instrument parameters set as above (Part 1).
 - b) Set up the autosampler and LC methods as described in WU-SOP-LC-1.

- c) Inject a (10 µl) 'HIGH' quality control sample containing equimolar H/L standard peptides (WU-SOP-EXP2-01) spiked in assay matrix and acquire the scheduled PRM-MS data. Perform acquisition on both LC columns.
- 4) Instrument performance evaluation with scheduled method.
- a) Import the data files into the Skyline document (Step 3A).
 - b) Check the automatic peak selection/integration of all peaks.
 - i. Manually adjust integration of peaks, if necessary. See Skyline tutorial, "Targeted Method Refinement" for reference. https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_method_refine
 - ii. Check integration by determining if start and stop are identical for all transitions of a precursor (go to "Settings", and check box "Integrate All" to enable this feature automatically).
 - c) Assess peak shape.
 - i. No tailing or fronting.
 - ii. No missing data (e.g. drop-out of electrospray).
 - iii. If the peaks are unacceptable, troubleshoot the LC system using the benchmark procedure described above and in WU-SOP-LC-1 and re-run the column conditioning procedure.
- a) Apply criteria for peak consistency between columns.
 - i. Retention time shift is < 2 min between columns.
 - ii. Peak intensities between columns is <20%.
- 1) Final method build.
- a) Save Skyline method test file as a final method.
 - b) Remove unscheduled DDAi (Step 3A) and method building scheduled DDAi data files (Step 3B) from the document (Edit>Manage Results). Save the document. The resulting Skyline document should now contain the most recent scheduled method testing data files with current retention times.
 - c) Export the scheduled precursor m/z list with averaged retention times as a report using above scheduled parameters.
 - d) Import the scheduled precursor m/z list as a scheduled DDAi method on the TTOF 5600+ using the Analyst® TF method wizard with all other parameters set as above (step 1).

E. Analysis of Response Curve For Characterization of Assays.

- 1) LC-PRM-MS method preparation
 - a) Load the Skyline file containing peptide sequences, precursors, transitions, and retention times to be monitored during the LC-PRM-MS experiment.

- b) In the Skyline file under Settings/Transition Settings/Predictions, select 'ABI TTOF 5600 under 'Collision energy:' and 'ABI' under 'Declustering potential'.
 - c) Export the scheduled precursor *m/z* list as a report in Skyline with a column for "Precursor Mz" and "Average Measured Retention Time" averaged using above scheduled parameters. See Skyline tutorial, "Custom & Live Reports" for reference. https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_custom_reports
 - d) Import the scheduled precursor *m/z* list as a scheduled DDAi method on the TTOF 5600+ using the Analyst® TF method wizard with all other instrument parameters set as above (Part 1).
 - e) Set up the autosampler and LC methods as described in WU SOP-LC1-01.
- 2) Experiment 1 or Experiment 2 .
- a) Refer to WU-SOP-EXP1-01 or WU-SOP-EXP2-01 for details on samples and queues.

8. REFERENCED DOCUMENTS

- A. WU-SOP-MS1-01
- B. WU-SOP-LC1-01
- C. WU-SOP-EXP1-01
- D. WU-SOP-EXP2-01
- E. Skyline manual and tutorials. <https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorials>

9. LIST OF ABBREVIATIONS

AcN, acetonitrile

FA, formic acid

LC-MS, *nano*-LC interfaced to a high-resolution quadrupole-time-of-flight mass spectrometer as described in WU-SOP-LC-1 and WU-SOP-MS-1

H or heavy, stable isotopically labeled synthetic peptide

L or light, natural abundance synthetic peptide

β-GAL, standard tryptic digest of β-galactosidase

Q.S., *quantum satis*

PDX, patient-derived xenografts

PRM, parallel reaction monitoring mass spectrometry

PS, primary stock solution; prepared by direct dilution and transfer from the vendor vials.

HSS, secondary stocks of the heavy primary peptide stock solution.

LSS, secondary stocks of the light primary peptide stock solution.